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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,262,056,** on February 24, 1999, by **UNIVERSITÉ DE MONTRÉAL,** assignee of Philippe Crine and Guy Boileau, for "Composition, Methods and Reagents for the Synthesis of a Soluble Form of Human Pex".

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ABSTRACT

This invention relates to a soluble form of PEX, PEX being a type II integral membrane glycoprotein. This enzyme is the gene product of a phosphate regulating gene with homologies to endopeptidases on the X chromosome. To produce a soluble form of PEX, the transmembrane anchor domain has been modified to encode a signal peptidase coding sequence. The soluble PEX therefore comprises the active ectodomain. An inactive mutant of PEX is also an object of this invention. Both soluble and inactive mutant forms of PEX can be used to screen ligands to PEX. These ligands will be used as substrates or inhibitors of PEX. PEX being phosphaturic, an inhibitor thereof will be used to treat phosphaturia and/or hypophosphatemia. On the opposite, a substrate for PEX or PEX itself will be used to treat hyperphosphatemia.

TITLE OF THE INVENTION

Composition, Methods and Reagents for the Synthesis of a Soluble Form of Human PEX

BACKGROUND OF THE INVENTION

The PEX gene (Phosphate regulating gene with homologies to Endopeptidases on the X chromosome) was identified by a positional cloning approach as the candidate gene for X-linked hypophosphatemia (XLH) (Francis et al., 1995). XLH is a Mendelian disorder of phosphate homeostasis characterized by growth retardation, rachitic and osteomalacic bone disease, hypophosphatemia, and renal defects in phosphate reabsorption and vitamin D metabolism (Rasmussen and Tenenhouse, 1995). Using the information made available by the publication of the sequence of the PEX gene, and standard techniques obvious to those in the art, several groups have cloned and sequenced the human and mouse PEX/Pex cDNAs (Du et al., 1996; Lipman et al., 1998; Grieff et al., 1997; Beck et al., 1997; Guo and Quarles, 1997; Strom et al., 1997). Amino acid sequence comparisons have demonstrated homologies between PEX/Pex and members of the neutral endopeptidase family as previously observed in the partial sequence of the candidate gene(Francis et al., 1995). The peptidases of the neutral endopeptidase family are type II integral membrane glycoproteins with a relatively short cytoplasmic N-terminal region, a single transmembrane domain, and a long extracytoplasmic domain, which contains the active site of the enzyme (Devault et al., 1987). Known members of the neutral endopeptidase family include neutral endopeptidase-24.11 (Neprylisin, NEP), endothelin-converting enzymes (ECEs), and the erythrocyte cell surface protein KELL (for a review see (Turner and Tanzawa, 1997b). NEP is a widely distributed peptidase involved in the degradation of several bioactive peptides, such as the enkephalins, the atrial natriuretic peptides, and the endothelins (Crine et al., 1997). The ECEs are involved in the bioactivation of big endothelins into endothelins (Turner, 1997a). No function has been yet attributed to Kell.

The mechanism by which loss of PEX function elicits the bone and renal abnormalities observed in XLH patients is not clear. There are no data suggesting the presence of PEX/Pex mRNA in the kidney (Du et al., 1996; Beck et al., 1997; Grieff et al., 1997). In contrast PEX/Pex mRNA was detected in bones by Northern blot hybridization and in other adult and fetal tissues such as lungs, liver, muscles, and ovaries by RT-PCR and RNase protection assays (Du et al., 1996; Beck et al., 1997). *In situ* hybridization performed on sections of embryos and newborn mice showed the presence of PEX mRNA in osteoblasts and odontoblasts (Ruchon et al., 1998). PEX gene expression was detectable on day 15 of embryonic development, which coincides with the beginning of intracellular matrix deposition in bones. Moreover, Northern analysis of total RNA from calvariae and teeth of 3-day-old and adult mice showed that

the abundance of the PEX transcript is decreased in adult bones and in non growing teeth. When the presence of the PEX protein in adult bones was investigated both by Western blotting, large amounts of immunoreactive protein were found. Immunohistochemical studies on a 2 month old mouse showed an extensive labeling of all cells of the osteoblast lineage including osteocytes. Taken together these results suggest that PEX plays an important role in the development and maintenance of mineralization in these tissues.

Further insights into the role of PEX in bone metabolism were provided by experimental studies on cases of oncogenic osteomalacia (OOM), a tumor-associated sporadic condition with very similar clinical presentations. There is strong evidence that a humoral factor produced by the tumor inhibits renal phosphate reabsorption and vitamin D synthesis resulting in osteomalacia (Nelson et al., 1997). Experimental studies on the Hyp and Gy mice, the murine model of human XL, also suggest the involvement of a humoral factor. In both mouse models, mutations have been identified in the PEX gene, which also appear to result in loss of function of the gene product (Strom et al., 1997; Beck et al., 1997). Considering the similarities between PEX and the other members of this metallopeptidase family, it has been speculated that PEX metabolizes a peptide hormone that modulates renal tubular phosphate reabsorption. Such an activity could involve either the processing of a phosphate reabsorbing hormone precursor to its active form or the inactivation of a circulating phosphaturic factor. There is evidence for intrinsic abnormalities in osteoblasts from Hyp mice (Ecarot et al., 1992). A defective phosphate transport was also observed in osteoblasts from Hyp mice (Rifas et al., 1994). PEX might thus be involved in the control of bone metabolism both indirectly at the level of the kidney by controlling renal phosphate reabsorption and directly at the level of bones by inactivating a trophic peptide factor controlling either osteoblast or osteoclast functions or both.

The identification and characterization of the putative PEX substrates will require first a better understanding of PEX function and enzymatic activity. Establishing the enzymatic activity of the enzyme and its substrate specificity will be greatly facilitated by having access to a pure preparation of the enzyme, free of other potential protease activities.

SUMMARY OF THE INVENTION

Towards this objective, we have prepared various reagents and tools designed to produce recombinant forms of PEX and purify both the recombinant and native enzymes from cell fractions, spent culture media and tissue extracts. We have cloned a cDNA encoding the full-length human PEX protein into various expression vectors. These PEX-encoding vectors were introduced by transfection into various cell lines including COS-1 (monkey kidney) cells, CHO (Chinese Hamster Ovary) cells, and LLC-

PK1 (porcine kidney) cells. Permanent cell lines were established and shown to stably express the PEX protein at the cell surface. A procedure was established to rapidly prepare a membrane fraction enriched in the recombinant PEX protein and this preparation was used to assess PEX enzymatic activity using bone-related peptides and growth factors.

PEX is an intrinsic membrane protein anchored by a hydrophobic 20 amino acid sequence located near the N-terminus. The purification of an intrinsic membrane-bound protein requires the use of detergents to free it from the lipidic environment of the membrane. These detergents can interfere with the catalytic activity of the enzyme. Moreover, the detergent-purified proteins usually present stability and solubility problems, especially if concentrated solutions and/or large amounts of the protein are needed, such as those required for crystallization and high throughput screening assays. To facilitate the preparation and purification in high yields of a fully active enzyme it is thus preferable to work with a soluble form of PEX. Soluble forms of NEP (Lemay et al., 1989) and ECE (Korth et al., 1997) consisting of the entire ectodomain but lacking the cytosolic and hydrophobic transmembrane domains have been constructed and shown to possess enzymatic activities identical to those of the native membrane-bound homolog. A soluble form of recombinant PEX was thus constructed by modification of the signal peptide/transmembrane region of the protein. The soluble PEX comprises PEX ectodomain or a catalytic part thereof. The expression vector encoding this soluble form of PEX was transfected into LLC-PK1 cells and a permanent cell line expressing the chimeric PEX protein on a stable basis was established. Analysis of the spent medium of this cell line by Western blot was shown to contain high levels of a soluble form of PEX.

Finally, monoclonal antibodies specific for PEX were generated by immunizing mice with a PEX-derived recombinant fusion protein produced in *E. coli*. These monoclonal antibodies were used to purify recombinant PEX by various immunoaffinity procedures. PEX-specific monoclonal antibodies were also proved useful for characterizing PEX expression in bone by immunohistochemical techniques and Western blotting.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS A OF THE INVENTION

BRIEF DESCRIPTIONS OF THE FIGURES

This invention will be described hereinbelow with reference to the following specific embodiments and drawings, which purpose is to illustrate the invention and not to limit its scope.

Figure 1: Construction of a soluble form of PEX. Figure 1A represents the schematic structure of the native membrane-bound form of the enzyme and the construct in which the POMC signal peptide has been fused in frame with the ectodomain of the native

enzyme. Figure 1B represents the construct where part of the sequence for the hydrophobic transmembrane (underlined) domain has been replaced by a more hydrophilic (C). In panel D, part of the hydrophilic sequence introduced in C has been deleted.

Figure 2: Amino acid sequence of human PEX. The boxed sequence represents the hydrophobic signal peptide/transmembrane domain. The underlined sequence represents the segment used for making the E. coli GST-fusion protein for monoclonal antibody production.

Figure 3: Screening of PEX monoclonal antibodies. Figure 3A: monoclonal antibodies were first selected for their capacity to bind the $PEX_{121-294}$ fragment produced in *E. coli* as tested by using the spent medium of hybridoma cultures in ELISA assays. Immunoglobulins from positive cultures were next tested for their ability to bind membrane-bound PEX from LLC-PK1 cells transfected with the PEX expression vector. Figure 3A is the Western blot analysis of LLC-PK1 extracts stained with the various hybridoma supernantants. Track 12 is the staining pattern obtained with PEX polyclonal antibody prepared in rabbit. Figure 3B: immunoprecipitation of a soluble form of PEX (secPEX). LLC-PK1 cells were first transfected with a vector encoding a soluble form of PEX as explained in the Material and Methods section. The spent medium of transfected LLC-PK1 cells was then used as a source of secPEX for immunoprecipitation experiments. The immunoprecipitation was performed by first saturating protein A Sepharose beads (Pharmacia) with a rabbit anti-mouse IgG fraction and then with the mouse immunoglobulins from the hybridoma supernatants selected as shown in Figure 3A. After washing, these beads were incubated in aliquots of the spent medium of LLC-PK1 cells producing secPEX (40µg of total protein). The beads were pelleted by centrifugation, washed and the presence of secPEX was assessed by boiling the proteins bound to protein A Sepharose in the electrophoresis sample buffer followed by Western blot analysis with a PEX polyclonal antibody. Track 8 shows the results of an immunoprecipitation done in the same conditions with a rabbit PEX polyclonal antiserum. Tracks 10 and 11 are control experiments prepared from mock transfected cells.

Figure 4: Expression of membrane-bound and soluble forms of recombinant PEX in COS-1 cells. COS-1 cells were transfected with SV-40 derived expression vectors containing either the entire coding sequence of PEX (Figure 4A) or a construct capable of promoting the secretion of the PEX ectodomain (see Methods) (Figure 4B). The cells were kept in culture for 16 h after transfection and either a membrane fraction (Figure 4A) or the spent medium (Figure 4B) were prepared as explained in Methods. The expression of PEX was monitored in Western blots with monoclonal antibody 15D7. As seen in Panel A a band migrating with a mobility corresponding to an apparent Mr of 105,000 was present in the membrane fraction of cells transfected with

the pCDNA3/RSV-PEX-FLB vector (lane 1). This band was absent from the extract of control cells (lane 2). Panel B shows the presence of a secreted soluble form of PEX in the spent medium of transfected cells, but not in control mock transfected cells.

METHODS

Expression of human PEX in transfected cells

A cDNA encoding for the full-length human PEX was obtained by Polymerase Chain Reaction (PCR) as previously described (Beck et al., 1997). The plasmid pCR2.1-PEX-FLB was generated by cloning this cDNA into pCR2.1 (Invitrogen). A restriction fragment (SpeI-EcoRV), which contained the entire PEX coding sequence, was digested, blunted, and subcloned into the mammalian expression vector (pCDNA3/RSV). The resulting plasmid (pCDNA3/RSV-PEX-FLB) contained the entire PEX cDNA under the control of the Rous Sarcoma Virus (RSV) promoter.

This recombinant vector was then expressed transiently in COS-1 cells by transfection. COS-1 cells were grown at 37°C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 5% COSMIC (Hiclone), 100 U/ml penicillin, and 100 μg/ml streptomycin. COS-1 cells were transfected using the calcium phosphate-DNA coprecipitation procedure. The day following transfection, the serum-containing medium was changed for a synthetic medium that consists of DMEM supplemented with 1 μg/ml BSA, 2.5 μg/ml insulin, 17.5 μg/ml transferrin, 2 μg/ml ethanolamine, 100 μg/ml soybean trypsin inhibitor and 10 μg/ml aprotinin. Finally, sodium butyrate was added to the synthetic medium, at a concentration of 10 mM, to enhance the expression of the plasmids carrying the RSV promoter. After 48 h, the cells were harvested and the membrane were prepared according to the procedure of (Korth et al., 1997).

The plasmid pCDNA3/RSV-PEX-FLB was also transfected in LLC-PK1 cells by the $CaPO_4$ precipitation method. Transfected cells were selected by adding 1% G-418 (v/v) to the medium. G-418 resistant cells were grown in rollers in medium 199 with Earle's salts, 2mM L-glutamine, Hepes and bicarbonate buffer supplemented with 5% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were grown up to confluence, for about a week, and harvested by scraping with a rubber policeman.

Construction and expression of a soluble form of recombinant PEX

To obtain a soluble form of recombinant human PEX, we first attempted to fuse in frame the cDNA encoding the signal sequence of a secreted protein (pro-opiomelanocortin or POMC) to the cDNA sequence of the ectodomain of human PEX (Figure 1, panel A). This strategy, which had successfully been used for other members of this family of peptidases, namely NEP and ECE (Lemay et al., 1989; Korth

et al., 1997), resulted in the production of a misfolded PEX protein that remained trapped in the rough endoplasmic of transfected cells. Therefore, an alternate strategy was developed consisting in the substitution of selected amino acids in the N-terminal hydrophobic membrane anchor of PEX to transform it into a cleavable signal sequence.

Transformation of the membrane anchor into a cleavable signal sequence for was carried out on the pCDNA3/RSV/PEX-FLB plasmid. Site-directed mutations (9 codons) and deletions (4 codons) were introduced by Polymerase Chain Reaction (PCR) amplification using oligonucleotide #5136 as the sense primer 5'CTGACAGTGATCGCTCAACAAACAACCAGTCAAGGTCTCTTAAGTCTCCAAG3' and oligonucleotide #5134 as the antisense primer 5'GGTTGTTTGTTGAGCGATCACTGTCAGGACAAACACGACCAGGGCAATTCG3' (Figure 1, panel B). The resulting plasmid, designated as to pCDNA3/RSV/PEX-MutE, encoded for a secreted form of PEX (secPEX).

This recombinant vector was then expressed transiently in COS-1 cells by transfection as described above. After 16 hours of incubation, the medium was recovered and concentrated by ultrafiltration (MW cut-off = 30 kDa) using a Centriprep cartridge (Amicon). To induce the stable expression of sec PEX in LLC-PK_1 cells, the plasmid pCDNA3/RSV-PEX-MutE was transfected in LLC-PK_1 cells by the $CaPO_4$ precipitation method. Transfected cells were selected by adding 400 µg/ml G-418 to the medium. G418 resistant cells were grown in rollers in medium 199 with Earle's salts, 2mM L-glutamine, 1mM sodium pyruvate, Hepes and bicarbonate buffer supplemented with 5% fetal bovine serum (FBS), 100 µg/ml G-418, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cells were grown up to confluence, for about a week. The day before harvesting, the serum-containing medium was changed for a synthetic medium that consists of DMEM supplemented with 1 µg/ml BSA, 2.5 µg/ml insulin, 17.5 µg/ml transferrin, 2 µg/ml ethanolamine, 100 µg/ml soybean trypsin inhibitor and 10 µg/ml aprotinin. Finally, sodium butyrate was added to the synthetic medium, at a concentration of 10 mM, to enhance the expression of the secPEX gene, which is under the control of the RSV promoter. After 16 hours of incubation, the medium was recovered and concentrated by cross-flow filtration (MW cut-off = 30 kDa) using a Sartocon Micro Unit (Sartorius).

Characterization of secPEX was done by immunoblotting. Briefly, proteins from the concentrated media were resolved on 7.5% SDS-PAGE, and transferred onto 0.45 µm nitrocellulose membranes. Membranes were incubated for one hour in TTBS (Tris Buffered Saline containing 0.05% Tween-20) supplemented with 5% (w/v) instant non-fat dry milk (Carnation). Membranes were washed rapidly with TTBS and incubated with a 1:200 dilution of the anti-(human PEX) monoclonal antibody (13B12) in TTBS supplemented with 1% BSA (w/v). Membranes were washed in TTBS and incubated

for one hour with a HRP-labeled second antibody in TTBS supplemented with 1% BSA (w/v). Membranes were washed and processed using a chemiluminescence reagent (NEN).

Purification of the soluble form of PEX

1) Purification of secPEX by immunoprecipitation:

The concentrated medium containing secPEX (40 µg/ml total protein) was diluted in 0.5 ml immunoprecipitation buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 2% sodium deoxycholate, 2%Triton X-100, 0.2% SDS, and 0.2% BSA) and polyclonal PEX antiserum was added. The solution was mixed for 12 hour at 4°C. By adding swollen protein A-Sepharose (Pharmacia) and by further mixing for 2 hour at 4°C, the immune complexes were precipitated. The beads were washed twice with immunoprecipitation buffer and once with PBS. The antigen bound to the immunoaffinity beads (secPEX) was recovered by boiling in the electrophoresis sample buffer and analyzed by SDS-PAGE followed by immunoblotting, as described above. Aliquots of the immunoprecipitate were kept at 4°C for enzymatic assays.

2) Purification of secPEX by ion-exchange chromatography:

The concentrated medium was supplemented with various protease inhibitors (100 µM phenylmethanesulfonyl fluoride, 20 µM pepstatin-A, and 20 µM leupeptin) and clarified by centrifugation (6,000g, 10 min, rotor Sorvall SS-34, 4°C). The clarified medium was loaded on a Q-sepharose anion-exchange column (Pharmacia) previously equilibrated with 20 mM Tris-HCl pH 8. Whereas most of the proteins bound to the column, secPEX did not bind to the resin at pH 8 and was recovered in the flow-through. The flow-through was concentrated by ultrafiltration (MW cut off = 30 kDa) using a Centriprep cartridge (Amicon) and diluted (1/10) with 50 mM ethanolamine-HCl pH 9.5. The flow-through was loaded on a Q-Sepharose anion-exchange column (Pharmacia) that was equilibrated with 20 mM ethanolamine-HCl pH 9.5. SecPEX was eluted with a 0 to 500 mM NaCl gradient and was analyzed by SDS-PAGE and immunoblotting, as described above. Alternatively, it is readily conceivable that SecPEX can be purified on an immunoaffinity column comprising an antibody specific to PEX.

Preparation of PEX-containing brush border membranes

The LLC-PK1 cell line forms polarized epithelial monolayers in culture. Brush border (apical) membranes BBMs were purified from LLC-PK1 cells homogenates as described previously in (Blais et al., 1987). Briefly, cell membranes were disrupted by sonication. Non-apical membranes were precipitated at 4 °C, under constant agitation, by adding $CaCl_2$ to a final concentration of 13 mM. BBMs were fractionated by sequential centrifugation at 950 x g for 10 min and then at 35, 000 x g for 30 min. The final pellet containing BBMs was washed twice with 50 mM Tris-HCl, pH 7.5, and

resuspended in the same buffer. The presence of PEX in BBMs was verified by immunoblotting.

RESULTS

Production of monoclonal antibodies

The cDNA corresponding to amino acids 121 to 294 of the PEX amino acid sequence (underlined segment in Figure 2) was used to construct a GST-fusion protein in E. coli. This fusion protein was purified from *E. coli* extracts by affinity chromatography on a glutathione-Sepharose column. After thrombin cleavage, the PEX portion of the GST fusion protein was further purified by electroelution from a polyacrylamide gel. This material was used to immunize 4 mice (5 injections of ≈50 μg of $PEX_{121-294}$). Blood was collected from each mice after the immunization schedule and the presence of antibodies in mice serum was assessed by ELISA using microtiter plates coated with $PEX_{121-294}$ from E. coli extracts. Mice sera were also tested for the presence of PEX antibodies by Western blotting extracts of LLC-PK1 cells transfected with the PEX expression vector. Out of the 4 mice immunized, 3 showed good results both in ELISA and Western blots. One mouse selected for its high titer of PEX specific antibodies (as measured by ELISA) was sacrificed and its spleen cells were collected and immortalized by fusion with myeloma cells(strain). Hybridoma cells were selected for their ability to grow in HAT selection medium and cloned by several rounds of limiting dilution. Throughout the limiting dilution process, hybridoma were tested for their ability to bind to $PEX_{121-294}$ in the ELISA assay and to recognize recombinant full length PEX in Western bloting assays (Figure 3A).

Construction of an immunoaffinity column

Hybridoma clones secreting immunoglobulins producing a strong signal in Western blotting (see above) were further submitted to additional screening assays designed to identify monoclonal antibodies capable of immunoprecipitating the soluble form of PEX in solution. The immunoprecipitation assay was performed by first saturating protein A Sepharose beads (Pharmacia) with a rabbit anti-mouse IgG fraction and then with the mouse immunoglobulins from hybridoma supernantants. After washing, these beads were incubated in aliquots of the spent medium of LLC-PK1 cells producing secPEX (40 μg of total protein). The beads were pelleted by centrifugation, washed and the presence of secPEX bound to the immunoaffinity support was assessed by submitting the proteins bound to proteins A Sepharose in a non-covalent fashion to booling in the electrophoresis sample buffer before immunoblot analysis (Figure 3B). Amongst the hybridoma analyzed for their production of specific anti-PEX antibody, the hybridoma 15D7 has been retained. It is understood that many monoclonal antibodies that have an equivalent immunological profile are under the scope of this invention.

Expression of membrane-bound recombinant PEX in COS-1 cells

COS-1 cells were transfected with an SV-40 derived expression vector containing the entire coding sequence of PEX inserted downstream from the RSV promoter. This vector is called pCDNA3/RSV-PEX-FLB (see Methods). The cell were kept in culture for 16 h after the transfection and a membrane fraction was prepared as explained in Methods. The expression of PEX was monitored in Western blots with monoclonal antibody 15D7. As seen in Figure 4 a band migrating with a mobility corresponding to an apparent Mr of 105,000 was observed in the membrane fraction of cells transfected with the pCDNA3/RSV-PEX-FLB vector (lane 1). This band was absent from the extract of control cells (lane 2). The mobility of this band was identical to that reported previously for recombinant human and mouse PEX.

Production, purification and characterization of a soluble form of recombinant PEX

We next wanted to determine whether it is possible to use genetic engineering techniques to promote the secretion of a soluble and active form of PEX from transfected eukaryotic cells. Obviously, this kind of enzyme, which can easily be purified from the incubation medium of cultured cells without the use of detergent would be very useful for further structural studies and inhibitor screening. It could also eventually be used as a injectable therapeutic agent or in topic applications to increase the rate of bone mineralization or bone healing.

PEX is a class II integral membrane protein. These membrane proteins have, near their amino terminus, a unique hydrophobic peptide acting both as a signal peptide to direct the translocation of the protein through the membrane of the rough endoplasmic reticulum and as a transmembrane domain for anchoring the protein in the cell plasma membrane. Unlike class I membrane proteins which possess a cleavable signal peptide and are anchored in the membrane by an additional membrane-spanning hydrophobic sequence (also called Stop Transfer Sequence), class II protein cannot be easily transformed into soluble forms by deleting the hydrophobic transmembrane domain. In class II proteins, deletion of the anchoring segment also removes the signal peptide, thereby preventing the translocation of the protein in the RER and its transport to the cell surface. Theoretically, there could be two different approaches for transforming a membrane-bound class II protein into a soluble form: 1) the extracellular domain of the protein could be fused to a heterologous cleavable signal peptide; 2) changes in the transmembrane domain could be introduced to transform the combined signal/anchor into a cleavable signal peptide. Both strategies were successfully used to produce a soluble from of NEP (Lemay et al., 1989; Lemire et al., 1997).

In this work, we first constructed a PEX secretion vector by fusing in-frame-the sequence encoding the complete ectodomain of the human enzyme with the POMC signal peptide (Figure 1A), these sequences being under the control of the RSV

promoter. Despite the fact that PEX immunoreactive material could be detected in the cell extract of transfected cells, expression levels were low and no enzyme could be found in the secretion medium. When the cell-associated PEX immunoreactive material was digested with endoglycosidases and analyzed by Western blot, it was found to be essentially endo H sensitive, indicating retention of the recombinant protein in the RER.

Replacement of part of the transmembrane region (underlined sequence in Figure 1B: sequence 1) by the underlined sequence shown on line 2 resulted in the secretion of a soluble form of PEX from transfected COS-1 cells. The yield was further increased by deleting the sequence LFLV at the junction between the transmembrane and ectodomain (panel B: sequence 3). Figure 4 shows the amount of recombinant protein secreted in the incubation medium by transfected COS-1 cells. The same vector was also transfected in LLC-PK1 cells as described in Methods and stable transfectants were selected for their G-418 resistance. This pool of G-418 resistant cells were found to secrete substantial amounts of secPEX (up to 100 μg/L) as seen by Western blotting. SecPEX was resistant to endo H, indicating that it has acquired terminal sugars most probably during its transit through the Golgi apparatus. The enzyme secreted by cultures of LLC-PK1 cells can then be purified either by immunoprecipitation or by ion-exchange chromatography, as explained in Methods.

EXAMPLES

Example I: Use of recombinant PEX to identify its natural substrate in bone

PEX is expressed at high levels in osteoblasts, and its expression is temporally associated with the mineralization of the extracellular matrix in cultured osteoblasts (Beck et al., 1997a; Du et al., 1996a; Guo and Quarles, 1997a) and during development (Ruchon et al., 1998a). These observations suggest that bone is a relevant site of PEX expression and that a potential relationship exists between mutations of PEX and aberrant osteoblast-mediated mineralization. Thus PEX may function in osteoblasts to metabolize endogenous or exogenous factors that regulate the process of osteoblast-mediated mineralization. In support of this hypothesis, a recent report suggests that abnormal PEX from cultured osteoblasts of Hyp mice is associated with the accumulation of a factor or factors that inhibit mineralization of extracellular matrix *in vitro* (Xiao et al., 1998). The availability of recombinant soluble PEX will greatly facilitate the identification of the physiological bone substrate(s) for PEX in a series of experiments such as the one described hereunder.

Bones of Hyp mice will be dissected, freed from connective tissue and muscles frozen in liquid nitrogen and lyophilized. The bones will then by crushed into a powder and extracted with a strongly acidic solution containing trifluoroacetic acid (TFA), formic acid and 1M NaCl. The composition of this solution will be selected such as to

inactivate all protease activities and avoid the solubilization of large molecular weight proteins. The acidic extract will then be lyophilized and an aliquot containing approximately 100 µg of total peptide resuspended in a physiological buffer at pH around 7.0, will be submitted to digestion with 1-10 µg of PEX purified by FPLC or by immunoaffinity chromatography as described above. A control experiment, where the enzyme preparation will be inactivated by acidic or heat treatment prior to the incubation will be conducted in parallel. The peptide contained in the samples will then be are separated by reversed-phase HPLC on a C18 µBondapak column using buffers containing 0.1% TFA and variable concentrations of acetonitrile (i.e from 0 to around 40%). The chromatograms of the peptide digested with active or inactivated PEX will be compared. The mixture of bone peptides taken from Hyp mouse and incubated with the inactivated PEX preparation should contain the PEX substrate. Incubation of the same mixture with active PEX however should allow the cleavage of the PEX substrate into peptide metabolites. Comparison of the chromatograms should thus allow to identify peaks corresponding to PEX substrate and its metabolites. These peaks will then be collected and identified by mass spectrometry and/or automated Edman sequence degradation.

The identification of PEX substrates could also be done using a similar strategy with conditioned medium taken from cultures of Hyp mouse osteoblasts.

Alternatively, an inactive soluble form of PEX immobilized on a chromatographic support could be used as an affinity reagent for purifying PEX substrates from crude extracts of tissues (such as bones) or serum. Cell surface metallopeptidases from the neprilysin family can me modified by the addition of a C-terminal extension without interfering with their enzymatic activity (Howell et al., 1995; Yang et al., 1995). An soluble form of PEX, extended by an additional C-terminal peptide of approximately 20-25 amino acid residues (called here secPEX-EC) will be constructed by fusing in frame a synthetic oligonucleotide as explained previously for NEP (Howell et al., 1995). The additional sequence will be terminated by a cysteine residue such as to allow its efficient coupling to activated thiol-Sepharose 4B [agarose-(glutathione-2-pyridyl disulfide)] (Pharmacia, Fine Chemicals AB, Uppsala, Sweden). Sec-PEX-EC, will be produced in high yields using for example but not exclusively, a Sf-9 baculovirus system as explained for NEP (Fossiez et al., 1992). The recombinant protein will be purified by column chromatography using conventional procedures. For example the spent medium of infected cell cultures will be concentrated and equilibrated with 20 mM Bis-Tris buffer pH 7 by centrifugation at 1500xg on Centriprep-30 cartridges (Amicon) at 4 °C. The concentrated culture medium will be loaded on a Resource Q ion-exchange chromatography column (Pharmacia) previously equilibrated with the same buffer. SecPEX-EC will be eluted from the column with a NaCl gradient from 0

to 0.5 M in 27.5 min at 4 ml/min. The fractions will be analyzed by SDS-PAGE and the purity verified by staining with Coomassie blue.

For binding the purified recombinant protein to the solid phase, the Thiol-Sepharose resin will be rehydrated to obtain approximately I ml of gel volume. The gel will equilibrated with a buffer A (0.1 M Bis-Tris, 0.5 M NaCl, pH 7.0) and incubated with approximately 3 mg of SecPEX-EC in buffer A (2-4 ml) overnight at 4 °C under constant agitation. The slurry will then be washed first with approximately I ml of buffer B (0.1 M Bis-Tris, 5 mM DTT, pH 7.0) and then extensively with buffer A. The quantity of proteins coupled to the support will be determined by the Bradford assay (BioRad) on a small amount of gel.

The immobilized SecPEX-EC will be used as a solid phase reagent for the screening of PEX inhibitors. Enzymatically inactive variants of this material will also be prepared by binding a form of SecPEX-EC carrying a mutation on the catalytic glutamic acid residue in position 582 to change it into a valine. A similar mutation in the coding sequence of NEP was previously shown to result in a catalytically inactive enzyme that nevertheless retained its full binding activity for inhibitors and substrates (Devault et al., 1988). Such an affinity reagent will be used to bind and purify PEX peptide substrates in crude tissue extracts. Receptors, if any, can be found using the same approach. Screening of inhibitor components can also be performed, although an active PEX may be preferred. Tissue extracts prepared as described above will be incubated under constant agitation in a buffer such as 0. IM Bis-Tris pH 7.5 with I ml of the affinity resin at 4°C. After washing in the same binding buffer, the bound peptides can be eluted from the gel by either raising or lowering the pH, and/or by increasing the ionic strength of the buffer.

<u>Example II:</u> Construction of an enzymatic assay

A peptide consisting for instance of 10 amino acid residues spanning the cleavage site of the natural peptide identified as explained in Example I, will be synthesized by solid-phase peptide synthesis and used as a substrate for PEX. This decapeptide (10 μg) will incubated in the presence of purified soluble PEX (1-10 μg total protein), at 37°C for 60 min. in Tris-HCl pH 7.5. The reaction will be terminated by the addition of lml of 0.1% TFA . Metabolites will be analyzed using a C-18 μ-Bondapack column (Waters). For example metabolites could be resolved with a 45 min linear gradient of 0-40% acetonitrile in 0.1% trifluoroacetic acid at a rate of 1.0 ml/min. The eluted peptides will detected by monitoring their absorbance at 214 and 254 nm. The decapeptide should be cleaved into two shorter peptides that will be eluted at different retention times. The peak fractions corresponding to these two peptides will be collected and their molecular mass will be determined by mass spectrometry to identify the position of the cleavage site. Once validated as a substrate for PEX, the synthetic peptide described here above will be modified such as to

incorporate amino acid derivatives bearing either fluorescent groups, chromogenic groups or radioactive atoms. These peptides derivatives will then be used to construct fast sensitive and robust enzymatic assays for further quantifying and characterizing PEX in tissue extracts as described in Example III.

Example III: Screening of inhibitors

For example, the peptide identified in Example II will be used to design and synthesize internally quenched fluorescent peptide substrates for PEX. Small peptide libraries are prepared with a fluorophore at one extremity and a quencher group at the other (Meldal, 1998). The substrate can be identified using a strategy described in (Apletalina et al., 1998). For each hexapeptide library, the identity of one residue at one position remains constant while the rest is randomized (for a total of 6*20=120 individual libraries). Each library is made-up of 3.2 million different members and is identified both by the position of the constant residue along the hexapeptide, and its identity. A purified preparation of PEX enzyme is added to each library and the fluorescence is recorded. The data is organized to identify the libraries producing the most fluorescence for each position along the hexapeptide. This arrangement suggests the identity of important residues at each position along the hexapeptide. Hexapeptide representing the best suggestions are prepared and tested in a similar fashion. From this set, the hexapeptide with the best fluorescence is selected. This assay can be useful for setting up a high throughput screening method for identifying inhibitors in combinatorial libraries of compounds.

Inhibitors can be identified from synthetic libraries, biota extracts and from rationally designed inhibitors using X-ray crystallography and substituent activity relationships. Each molecule or extract fraction is tested for inhibitory activity using the enzymatic test described above. The molecule responsible for the largest inhibition is further tested to determine its pharmacological and toxicological properties following known procedures. The inhibitor with the best distribution, pharmacological action combined with low toxicity will be selected for drug manufacturing. Pharmaceutically acceptable formulation of the inhibitor or its acceptable salt will be prepared by mixing with known excipients to produce tablets, capsules or injectable solutions. Between l and 500 mg of the drug is administered to the patients;

Example IV: Uses of recombinant PEX protein in therapeutic applications

The murine Hyp model reproduces the characteristics of human X-linked hypophosphatemia (XLH), an inherited disease causing renal loss of phosphate (Pi), severe rickets and osteomalacia. The presence of renal phosphate wasting secondary to a mutation in the PEX gene suggests that this endopeptidase degrades a yet unidentified phosphaturic hormone, referred to as phosphatonin (Kumar, 1997). To test this hypothesis directly, we will prepare primary mouse proximal tubule cell cultures (MPTC), expressing normal features of proximal tubule cells. The presence of 10%

Hyp mouse serum in HAMF12/DMEM media (1 mM Pi) for the last 48 hours of culture of MPTC was previously found to reduce Pi uptake by 45.7 +/- 3.9% as compared to normal mouse serum in a dose- and time-dependent manner (Lajeunesse et al., 1996). If defects in the PEX gene in Hyp mouse osteoblasts, is responsible for the release and/or the modification of a factor that can reach the circulation and which inhibits renal phosphate reabsorption, it would be possible to abolish the effect of the Hyp mouse serum on Pi uptake by pretreating the serum with a purified preparation of PEX. The effect of PEX (1-10 µg of purified recombinant soluble PEX) on Hyp mouse serum will then be monitored by measuring phosphate uptake by MPTC cells. Control experiments will include incubating the serum samples under similar conditions but with heat or acid inactivated PEX. If PEX treatment is found to restore normal phosphate uptake, recombinant soluble PEX might thus be used as a therapeutic agents for restoring normal phosphate levels first in animal models (such as the Hyp mouse or experimental models of chronic renal failure) and then in patients with pathological states characterized with chronic renal failure. These patients develop hyperphosphatemia that causes a number of complications such as ectopic calcifications and secondary hyperparathyroidism. This last complication inevitably leads to metabolic bone diseases and increased morbidity and mortality. In these patients, recombinant soluble PEX given for example but not exclusively, as an intravenously injectable drug could help lower circulating phosphate levels and thus alleviate the problems associated with hyperphosphatemia.

Example V: Production and use of PEX antibodies

As shown in the present work, knowledge of PEX cDNA sequences can be used to raise specific antibodies. For example but not exclusively, regions of less homology between the peptidases (amino acid residues 121 to 294) can be used to synthesize peptides whose sequences are deduced from the translation of the cDNAs, and/or bacterially-expressed fragments of the cDNAs fused for example but not exclusively to GST may be purified and injected into rabbits or mice for polyclonal or monoclonal antibody production. These antibodies can be used to:

- identify by immunohistochemistry the peptidergic pathways in which the peptidases are functioning;
- study the physiopathology of PEX by immunoblotting or immunohistochemistry on samples of biological fluids or biopsies;
- set up high through put screening assays to identify PEX inhibitors. This can be done for example but not exclusively by using the antibodies to attach the PEX to a solid support;
- purify PEX with said antibodies by immunoprecipitation or affinity chromatography by identifying antibodies capable of selectively binding to PEX in one set of conditions and releasing it in another set of

conditions typically involving a large pH or salt concentration change without denaturing the PEX enzyme;

- identify antibodies that block PEX activity and use them as therapeutic agents. Blocking antibodies can be identified by adding antisera or ascite fluid to an *in vitro* enzymatic assay as described in Example II and looking for inhibition of NL-enzymes activities. Blocking antibodies could then be injected to normal or disease model animals to test for *in vivo* effects.

Example VI: Alternative methods for producing recombinant soluble PEX enzymes

As shown above, recombinant active PEX enzymes can be obtained by expression of PEX cDNAs in mammalian cells. From past experience with neprilysin, another member of the family (Devault et al., 1988; Fossiez et al., 1992; Ellefsen et al., 1998), expression can also be performed in other expression systems after cloning of PEX cDNA in appropriate expression vectors. These expression systems may include but not exclusively the baculovirus/insect cells or larvae system and the Pichia pastoris-based yeast system. Production of recombinant PEX enzymes includes the production of naturally occurring membrane bound or soluble forms of the protein or genetically engineered soluble forms of the enzyme. The latter can be obtained by substituting the cytosolic and trans-membrane domain by a cleavable signal peptide such as that of proopiomelanocortin, but not exclusively, as done previously (Lemay et al., 1989a) or by transforming by genetic manipulations the non-cleavable signal peptide membrane anchor domain into a cleavable signal peptide, as done previously (Lemire et al., 1997a) or by fusion of the ectodomain of PEX enzyme to the amino-terminal domain (from the initiator methionine to amino acid residue 300) of naturally occurring soluble NEP-like enzyme such as, but not exclusively, NL- I as done in other work.

EXAMPLE VII: Treatment of hypo-and hyper phosphatemic diseases

OHO mouse model is a hypophosphatemic disease model. This disease is correlated with an overexpression of PEX. Therefore, the administration of an anti-PEX molecule would be expected to normalize the symptoms. So, the administration of effective amount of PEX inhibitors or neutralizing antibodies formulated in a pharmaceutical compositions will expectedly result in treating diseases wherein overproduction of PEX occurs. Clinical results obtained with OHO model should validate in humans. On the opposite, the soluble PEX enzyme will be used to treat hyperphosphatemic diseases.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such

departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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We claim:

1. A soluble purified human PEX enzyme.

2. A mutant of the enzyme of claim 1, which is inactive but retains binding capacity to a ligand molecule to PEX.

3. A mutant as defined in claim 2 essentially consisting of PEX enzyme having the glutamic acid residue at position 582 substituted with a valine residue.

4. A nucleic acid encoding the enzyme of claim 1, 2 or 3, which comprises a truncated PEX gene sequence encoding PEX membrane-anchor domain modified to include a cleavable signal peptide and PEX C-terminal ectodomain.

5. A nucleic acid as defined in claim 4, wherein said cleavable signal peptide is pro-opiomelanocortin signal peptide.

6. A recombinant vector comprising the nucleic acid of claim 4 or 5.

7. A recombinant vector as defined in claim 6, which is an expression vector.

8. A recombinant host comprising the recombinant vector of claim 6.

9. A recombinant host comprising the recombinant vector of claim 7.

10. A method for producing a soluble PEX enzyme or an inactive mutant thereof, which comprises the steps of:

   - culturing the recombinant host of claim 9 in a medium supporting cellular growth and expression of said nucleic acid, and
   - recovering the soluble PEX enzyme or mutant thereof from the medium.

11. An antigenic composition which comprises the enzyme of claim 1.

12. An antibody raised against the enzyme of claim 1, or fragment thereof capable of binding to PEX.

13. An antibody as defined in claim 12 which is a monoclonal antibody.

14. The antibody of claim 13 which is a PEX neutralizing antibody.

15. A hybridoma producing the antibody of claim 13 or 14.

16. A pharmaceutical composition comprising the enzyme of claim 1 or the antibody of claim 14 as an active ingredient and a pharmaceutically acceptable carrier.

17. A kit for detecting the presence and/or amount of PEX comprising the antibody of claim 12 or 13.

18. A device for purifying the PEX enzyme or mutant thereof defined in claim 1, 2 or 3 which comprises an antibody directed against said enzyme or mutant, which antibody is fixed onto a solid support.

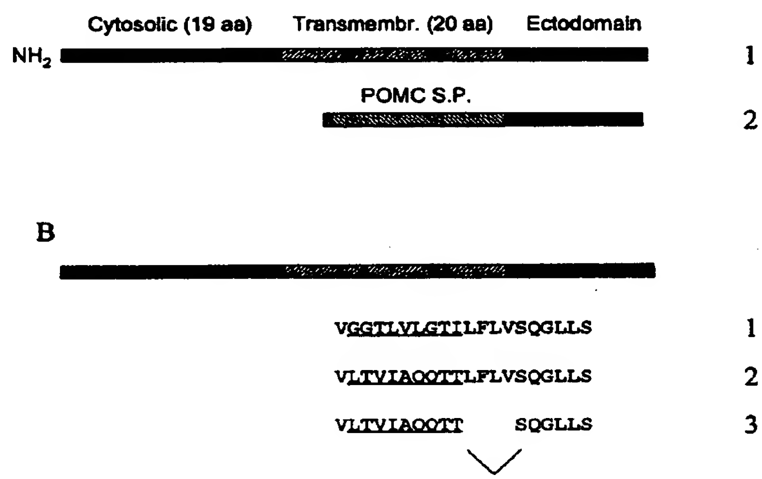
19. A device for screening PEX ligands, which comprises the soluble PEX enzyme or mutant thereof of claim 1, 2 or 3 fixed onto a solid support.

20. The device of claim 18, wherein PEX enzyme or mutant thereof is fixed onto the solid support through its binding to an anti-PEX antibody itself fixed onto said solid support.

21. The device of claim 18, wherein said PEX enzyme or mutant thereof is fixed through a C-terminal amino acid extension ending with a residue or group capable of coupling PEX to said solid support.

Figure 1

A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Ectodomain
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POMC S.P.
2
B
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VLTVIAQQTTLFLVSQGLLS
2
VLTVIAQQTT SQGLLS
3

Figure 2

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  1 MEAETGSSVE TGKKANRGTR IALYVFYGGL LVIGTILFLV SQGLLSLQAK QEYCLKPECI
 61 EAAAAILSKV NLSVDPCDNF FRFACDGWIS NNPIPEDMPS YGVYPWLRHN VDLKLKELLE
121 KSISRRRDTE AIQKAKILYS SCMNEKAIEK ADAKPLLHIL RHSPFRWPVL ESNIGPEGVW
181 SERKFSLLQT LATFRGQYSN SVFIRLYVSP DDKASNEHIL KLDQATLSLA VREDYLDNST
241 EAKSYRDALY KFMVDTAVLL GANSSRAEHD MKSVLRLEIK IAEIMIPHEN RTSEAMYNKM
301 NISELSAMIP QFDWLGYIKK VIDTRLYPHL KDISPSENVV VRVPQYFKDL FRILGSERKK
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421 MFVDVYFQED KKEMMEELVE GVRWAFIDML EKENEWMDAG TKRKAKEKAR AVLAKVGYPE
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541 YSASTNQIRF PAGELQKPFF WGTEYPRSLS YGAIGVIVGH EFTHGFDNNG RKYDKNGNLD
601 PWWSTESEEK FKEKTKCMIN QYSNYYWKKA GLNVKGKRTL GENIADNGGL REAFRAYRKW
661 INDRRQGLEE PLLPGITFTN NQLFFLSYAH VRCNSYRPEA AREQVQIGAH SPPQFRVNGA
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Figure 3

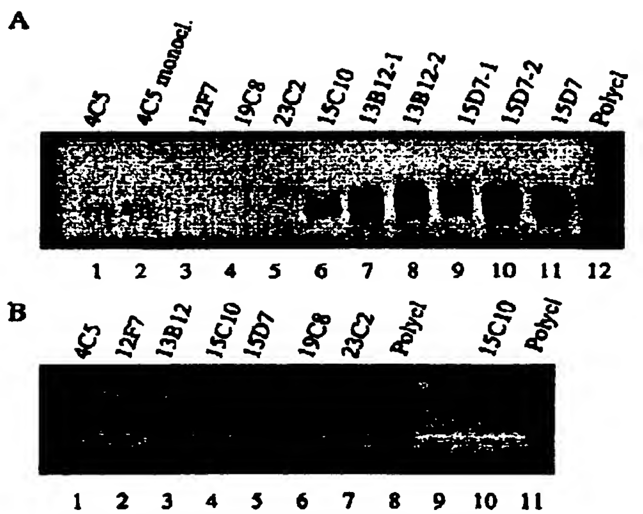
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Figure 4